

A BIOSENSOR METHOD FOR A COMPETITIVE IMMUNOASSAY DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN B (SEB) IN MILK¹

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ABSTRACT

A sensitive and more rapid biosensor method for the detection of staphylococcal enterotoxins (SE) is needed by the food industry. Staphylococcal enterotoxin B (SEB) is highly heat resistant and is a potential bioterrorism agent. Our research objective was to develop a competitive immunoassay using a surface plasmon resonance (SPR) biosensor for the detection of SEB below 1 ng/mL [part per billion (ppb)] in fresh fluid milk. The assay consisted of SEB immobilization on the sensor surface. An anti-SEB was allowed to bind with the SEB in samples off line prior to the biosensor analysis. The excess and unbound anti-SEB was then captured by SEB sensor. The assay conditions were optimized to detect SEB in HEPES buffer and in whole milk. An analysis of milk samples spiked with 0.312–50 ppb. SEB consisted of heating the samples at 95°C followed by rapid cooling and centrifugation at 2961 × g to separate the skim fraction. Aliquots of the skim fraction containing SEB were allowed to bind with anti-SEB for 30 or 60 min. The SEB and anti-SEB complex were separated from the free anti-SEB by centrifugation, and the supernatants were injected over the sensor. SEB was detectable in buffer at 0.78–50 ppb and in spiked whole and skim milk from 0.312–25 ppb. The biosensor analysis including the sensor regeneration was 15 min per sample in a fully automated system. The competitive assay format resulted in higher detection sensitivity and greater sample throughput than the SPR biosensor sandwich assay. The competitive assay will be utilized for the detection of SEB

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in various foods and will be optimized for the detection of other staphylococcal toxins in foods.

INTRODUCTION

The food industry needs routine methods to detect trace levels of enterotoxins produced by *Staphylococcus aureus*. *Staphylococcus aureus* produces enteric toxins that cause a major food-borne gastroenteritis. Under temperature-abused conditions, the organism can grow in foods of animal origin. Heat processing and normal cooking temperatures can inactivate or kill the bacterial cells, but staphylococcal enterotoxins (SEs) are heat stable and resistant to cooking and heating temperatures (Bergdoll 1979; Newsome 1988). Ten SEs have been identified: A, B, C1, C2, C3, D and E (Bergdoll 1979); H (Su and Wong 1995); I, G (Munson *et al.* 1998) and J (Zhang *et al.* 1998). SE A, B, C and D are the most common in foods, and staphylococcal enterotoxin A (SEA) is frequently recovered from food poisoning outbreaks.

Mead *et al.* (1999) reported an estimated 185,000 cases of staphylococcal food poisoning. The Centers for Disease Control and Prevention (CDC) reported 4870 cases in a passive surveillance from 1992 to 1997, and 487 cases were reported from outbreaks. In all cases, the toxins were transmitted through food. Staphylococcal enterotoxin B (SEB) is a potent GI toxin and is heat resistant. SEB is also a potential bioterrorism agent (CDC 2000). Staphylococcal enterotoxin E (SEE) has the least occurrence in outbreaks. The toxins have molecular weights (MWs) ranging from 27,000 to 34,000, and SEB has an MW of 28,336 with an isoelectric point (pI) of 8.6 (Jones and Khan 1986). The relative thermostability of these toxins is staphylococcal enterotoxin C (SEC) > SEB > SEA (Tibana *et al.* 1987), and the chemical and physical properties of these toxins are summarized by Jay (2000). The minimum level of enterotoxin to cause gastroenteritis in humans was approximately 1 ng/g or ng/mL of food (Noletto and Bergdoll 1982; Jay 2000), while Newsome (1988) and Tatini *et al.* (1984) reported less than 1 µg of toxin ingestion. Methods with detection at or below 1 ng/g [1 part per billion (ppb)] are desired, and testing for both the organism and toxin can assure the safety of processed foods.

Immunochemical assays were developed and utilized in the last 20 years for the detection of SE. The principles and effectiveness of these methods are described by Bergdoll (1996), Park *et al.* (1992, 1994) and Wieneke (1991). Su and Wong (1997) also reviewed the biological, immunological and polymerase chain reaction- (PCR)-based methods for the detection of SE. Biosensor techniques offer a rapid, automated and multi-toxin approach to detect these toxins in a food matrix. The principles and applications of the bioaffin-

ity-based sensors and the surface plasmon resonance (SPR) biosensors have been described and reviewed by Hodgson (1994), Malmquist and Karlsson (1997), Fivash *et al.* (1998), Nice and Catimel (1999) and Rich and Myska (2000). Medina (1997) described the application of the SPR biosensor in the analysis of foods. The BIAcore, an SPR biosensor, allows direct real-time detection of the binding without chemically altering the structures of the ligands or analytes to generate signals. Kinetic properties such as association and dissociation rates of the binding reactions of the binding ligands and analytes can be determined. A capturing molecule is covalently immobilized to the sensor chip and the binding molecule is captured by the immobilized ligand in a continuous flow system. The mass of the captured molecule generates a change of the refractive index of the medium in the vicinity of the sensor. These changes are then detected by an optical system that measures the intensity and angle of the reflected light. These interactions are expressed in arbitrary response units (RU) which are continuously monitored and are plotted in real time as RU versus time (s).

The SPR biosensors have been utilized for the detection of SE. A method for the detection of SEA spiked in milk, hotdogs, mushrooms and potato salad with an IAsys SPR biosensor (Affinity Sensors, Paramus, NJ) had a sensitivity of 10–100 ng/g (Rasooly and Rasooly 1999). The BIAcore SPR biosensor was also utilized to detect SEB spiked in potted meat at 10–1000 ng/g and 1–1000 ng/mL in reconstituted dry milk with minimum detection of 10 ppb (Rasooly 2001). This method utilized a 100- μ L sample in a flow rate of 20 μ L/min, allowing a 5-min contact time. Likewise, Medina (2003) reported the detection of 2.5 ppb SEB-spiked ham tissues using the BIAcore biosensor. Nedelkov *et al.* (2000) reported the detection and confirmation of SEB and toxic shock syndrome toxin-1 (TSST-1) with the BIAcore SPR biosensor and mass spectrometry. The latter identified the bound toxins by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) spectrometry. The SEB spiked in milk and mushrooms was detected at 1 ng/mL in milk and mushroom extracts that had been diluted 100 times. This method detected SEB at 100 ppb of the original sample concentration. Naimushin *et al.* (2002) reported the development of a miniature integrated two-channel SPR biosensor and detected 1 nM (28.4 μ g/mL) SEB in sea water and 50 pM (1.42 μ g/mL) SEB in urine. Homola *et al.* (2002) developed a modulation-based SPR biosensor and showed the detection of 5 ng/mL SEB. With a sandwich assay detection mode, the lowest detection limit was 0.5 ng/mL in buffer and milk samples. A particle-based automated fluorometric immunosensor technique detected 5 ng/mL SEB in cream in a sandwich assay format (Strachan *et al.* 1997). An automated optical flow cell with three-channel immunosensor detected 10 ng/mL of SEB in phosphate buffer with simultaneous detection of *E. coli* and M13 bacteriophage (Koch *et al.* 2000). King *et al.* (1999)

reported SEB detection at 100 ng/mL in 1% solutions of clay, topsoil and pollen using a fiber optic biosensor, the Man-Portable Analyte Identification System (MANTIS) developed by Research International (Woodinville, WA).

The objectives of the current study were to utilize a biosensor for improved detection of SEB to detect below 1 ng/mL, optimize the detection of SEB in a competitive inhibition assay format, optimize sample preparation of fluid milk for the biosensor analysis of SEB and improve the analytical throughput for routine analysis. The optimized method will be adopted for SEB detection in other food matrices and will also be used for the detection of SEA. Our long-term goal is to develop a multi-toxin detection of staphylococcal toxins in foods.

MATERIALS AND METHODS

Equipment and Reagents

The BIAcore upgraded with BIAcore 1000 system software was equipped with BIAevaluation 2.1; the CM-5 and Pioneer Chip F1 sensor chips, surfactant P20, amine coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC), ethanolamine and BIAcore sample tubes were from BIAcore, Inc. (Piscataway, NJ). SEB and the affinity purified polyclonal sheep antibody against SEB were obtained from Toxin Technology (Sarasota, FL). The Tomy Refrigerated Microcentrifuge MTX 150 was from Peninsula Laboratories (Belmont, CA), while the multi-block heater was from Laboratory-Line Industries, Inc. (Melrose Park, IL). HEPES (free acid), sodium azide (NaN_3), ethylenediamine tetraacetic acid (EDTA), sodium hydroxide (NaOH) and sheep anti-SEB serum were from Sigma Chemical Company (St. Louis, MO). The anti-SEB from Sigma was purified in our laboratory using an ImmunoPure Protein G Plus affinity column (Pierce, Rockford, IL). The milk samples were purchased from local markets.

Surface Plasmon Resonance BIAcore Analysis

These studies were performed on an upgraded BIAcore 1000 equipped with BIAlogue command software. The guidelines were followed for programming the methods, preparation of the sensor surfaces, binding techniques and interpretation of the sensorgrams (BIAcore Handbook 1994; Introduction to BIAcore 1994). The results from the real-time interactions of the ligand and the capturing molecule of the sensor were displayed in a sensorgram as optical RU versus time (s). The BIAevaluation 2.1 software was utilized for affinity and kinetic evaluation such as association or "on rate" and dissociation or "off" rate.

Preparation of the Sensor

SEB Sensor. The SEB toxin was diluted with 10 mM NaAc, pH 4.5, to a concentration of 0.5 mg/mL. A 200- μ L aliquot of SEB toxin was transferred to the BIAcore sample tube and placed in the sample rack. The immobilization started with the conversion of the carboxymethyl groups on the dextran surface of the flow cells on the sensor chips by activation with 10 μ L of a mixture of equal volumes of NHS (115 mg/mL) and EDC (750 mg/mL). The carboxyl groups were converted to NHS ester with EDC-NHS, and these esters spontaneously reacted with the uncharged amino groups that are favored by a pH below the pKa or pI of the ligand. A 30- μ L aliquot of the SEB preparation was automatically injected over the activated dextran. The remaining activated esters (not covalently bonded with the IgG or toxin) on the dextran surface were inactivated (blocked) with 30- μ L ethanolamine. The ligands and ethanolamine were injected at a flow rate of 3 μ L/min. HEPES-buffered saline (HBS), pH 7.4, containing 10 mM HEPES (free acid), 3.4 mM EDTA, 0.15 M sodium chloride (NaCl) and 0.005% BIAcore P20 surfactant (v/v), was utilized as the running buffer.

Preparation of SEB Standards and Anti-SEB

The SEB toxin was diluted to 1 mg/mL with deionized water, aliquoted at 100 μ L and stored at -80°C (stock A). A 100- μ L aliquot of 1 mg/mL SEB was diluted with the HBS (pH 7.4) to 1 mL to a final concentration of 10 μ g/mL (stock B) and further aliquoted to 100 μ L each. The working dilution of 100 ng/mL (ppb) SEB was prepared by diluting 100 μ L of stock B with 9.9 mL HBS. The SEB working standards (stock C) were serially diluted with HBS (pH 7.4) from 100 to 1.56 ng/mL (ppb). Later experiments used standards by serially diluting 50–0.78 ng/mL, 25–0.39 ng/mL and 20–0.31 ng/mL.

Characterization of the SEB Sensor

The HBS (pH 7.4) was utilized as the running buffer. This pH allowed the protonation of the SEB toxin, which has a pI of 8.6. (Jones and Khan 1986). The binding characteristics of the sensors were evaluated with various concentrations of anti-SEB (25, 50, 100; 12.5, 25, 50 and 2.5, 5 and 10 μ g/mL). The HBS (200 μ L) was transferred to the BIAcore sample tubes, and 20 μ L of the anti-SEB dilution was added. The antibody solutions were mixed and the tubes were transferred to the BIAcore sample rack. The analysis consisted of an injection of 15 μ L of the antibody mixture at a flow rate of 3 μ L/min. The bound complex was desorbed from the SEB sensor with 5 μ L of 20-mM NaOH at a flow rate of 5 μ L/min. The kinetic evaluation (“on and

off rates” and affinity constants) of the anti-SEB was determined from the capture of the 20 μL of 50 $\mu\text{g}/\text{mL}$ (333.3 nM) anti-SEB added to 200 μL HBS. The resulting final IgG concentration was 33.3 nM. Furthermore, the competitive inhibition responses of the 25 and 50 $\mu\text{g}/\text{mL}$ concentrations were compared when added to SEB in HBS or milk samples.

Competitive Inhibition Assay in HBS

This assay format was utilized to increase the sensitivity of the SEB assay by capturing the anti-SEB on the SEB toxin sensor. Serial dilutions of 5–100, 1–50 or 0.78–50 ppb SEB were prepared as described in the previous paragraph. The HBS samples (200 μL) containing SEB were transferred to the BIAcore sample tubes, followed by the addition of 20 μL anti-SEB (50 or 100 $\mu\text{g}/\text{mL}$) to each tube except for an HBS control tube. The samples were mixed off line and allowed to incubate for 20–30 min, and then were analyzed with the BIAcore without separating the bound complex. The injection of 15- μL samples (3 $\mu\text{L}/\text{min}$ flow rate) over the toxin sensor surface allowed the SEB toxin sensor to capture the excess antibody in the samples. The bound anti-SEB was measured 60 s after injection. The sensor surface was regenerated by desorbing with one pulse of 5 μL of 100 mM hydrochloric acid (HCl) at a 5 $\mu\text{L}/\text{min}$ flow rate. The resulting RUs were plotted against the SEB concentration. The principles of the competitive immunoassay are shown in Fig. 1. Our early studies showed variable responses compared with the sand-

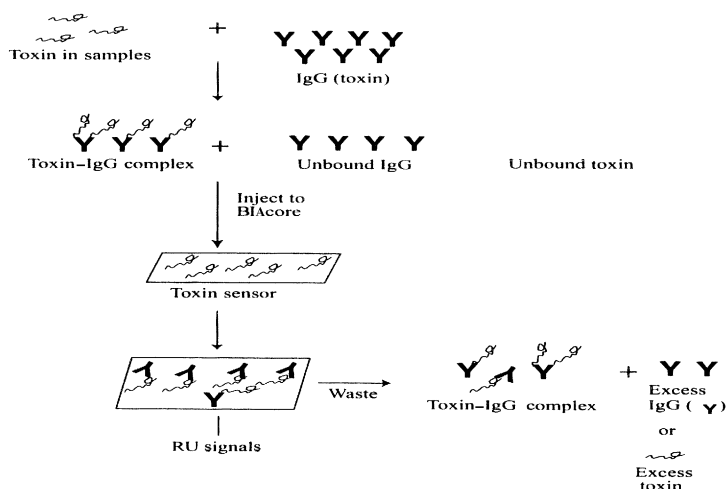


FIG. 1. FLOWCHART OF THE PRINCIPLES OF THE BIOSENSOR COMPETITIVE IMMUNOASSAY FOR THE DETECTION OF SEB BACTERIAL TOXIN

wich immunoassay (Medina 2003), and to improve the assay reliability, the SEB-IgG bound complex was separated from the excess antibody by centrifugation at 6000 or 10,000 r.p.m. (2961 or $8225 \times g$), respectively.

SEB Analysis in Whole Milk

Whole milk (1 mL) was transferred to 4-mL conical tubes, and 100 μL of 0, 3.125–200 ppb SEB standards was added. The final concentration of SEB added to the milk samples was 0, 0.312–20 ng/mL. The HBS and milk blank samples were used as control samples. The samples were mixed and allowed to equilibrate for 30 min or overnight at 4C. The milk sample tubes were then placed in a heating block for 15 min at 95C where the milk samples were held at 95C for 10 min. The samples were cooled in an ice bath prior to centrifugation at 6000 rpm ($2961 \times g$) for 10 min. The samples were mixed after every step. The aqueous fractions (skim supernatants) were pipetted into another set of microfuge tubes, and 200- μL aliquots were transferred to the BIAcore tubes. An anti-SEB (20 μL of 50 $\mu\text{g/mL}$) was added to each sample tube (except the HBS and milk blank samples) and allowed to bind with the SEB in milk for 1 h at room temperature. The SEB-anti-SEB complex was separated by centrifugation at 6000 r.p.m. for 10 min, and the supernatant of each sample was directly analyzed with the BIAcore system. The injection of 15- μL samples (3 $\mu\text{L/min}$ flow rate) over the toxin sensor surface allowed the SEB toxin sensor to capture the excess antibody in the samples. The bound anti-SEB was measured 60 s after injection. The sensor surface was regenerated by desorbing with 1–2 pulses of 5 μL of 100 mM HCl, 10 mM NaOH or 20 mM NaOH at a 5 $\mu\text{L/min}$ flow rate.

SEB Analysis in Skim Milk

Skim milk (1 mL) was transferred to 4-mL conical tubes, and 100 μL of 0, 3.125–200 ppb SEB standards was added to a final concentration of 0, 0.312–20 ng/mL. HBS and skim milk blank samples were used as control samples. The samples were mixed and allowed to equilibrate overnight at 4C. The milk samples were then placed in a heating block for 15 min at 95C where the samples were held at 95C for 10 min. The samples were cooled in an ice bath, and 200 μL -aliquots were transferred to the BIAcore tubes. An anti-SEB (20 μL of 50 $\mu\text{g/mL}$) was added to each sample tube (except the HBS and milk blank samples) and allowed to bind with SEB in milk for 1 h at room temperature. The samples were mixed every 20 min. The SEB-anti-SEB complex was separated by centrifugation at 6000 r.p.m. for 10 min, and the supernatant of each sample was directly analyzed with the BIAcore system. The injection of 9- μL samples (3 $\mu\text{L/min}$ flow rate) over the toxin sensor surface allowed the SEB toxin sensor to capture the excess antibody in the

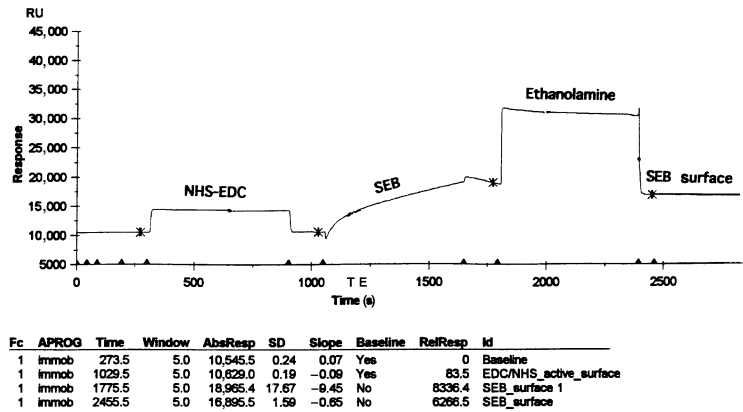


FIG. 2. PREPARATION OF SEB SENSOR

Sensorgram of SEB immobilization on Flow Cell 1 (FC 1) of the Pioneer F1 Chip (also CM3). The carboxyl groups of the dextran surface were converted to NHS esters, which covalently bound to the free amino groups of the SEB. The residual active esters were blocked with ethanolamine and the resulting SEB surface had a net RU of 6266, equivalent to 33.147 ng SEB/mm². (Sensor surface = 6266 RU/1000 = 6.266 ng equivalent IgG × 150,000 IgG MW/28,366 SEB MW.) The SPR biosensor detected the “real time” change (s) of the refractive index (indicated by RU) resulting from the binding of SEB molecules to the dextran surface.

samples. The bound anti-SEB was measured 60 s after injection. The sensor surface was regenerated by desorbing with one pulse of 30 mM NaOH at a 5 µL/min flow rate (Fig. 2).

SEB Sensor Surface

The carboxyl groups on the dextran surface of the BIAcore CM5 and Pioneer F1 (BIAcore CM3) sensor chips were activated with NHS and EDC for the covalent linkage of the SEB. The excess NHS–ester active sites were blocked with ethanolamine. The resulting surface load of the immobilized SEB ligands is shown in Table 1. The sensor surface protein load of 1 ng IgG/mm² generated 1000 RU of SPR signal (Karlsson *et al.* 1991; Fagerstram and O’Shannessy 1993). The Pioneer Chip F1 has shorter dextran surface molecules compared to the CM5 standard chip. Table 1 shows the immobilization load of 0.5 mg/mL SEB. F1 #91/FC 3 and F1 #100/FC 4 sensors were modified with 1 mg/mL and 0.25 mg/mL SEB, respectively. The immobilization contact time was 10 min (30 µL at 3 µL/min). The SEB surface (ng/mm²) was determined as: RU SEB divided by 1000 × 5.29 factor. This factor was a ratio of the MW of IgG to SEB (150,000/28,336 = 5.29). A 1000-RU surface resulting from the immobilization was equivalent to 1 ng IgG/mm². The mean

TABLE 1.
 SEB PROTEIN LOAD ON THE SPR SENSOR CHIPS

Chip number/ FC number	Immobilized SEB RU	ng SEB/mm ² *	RU IgG bound, 5 μ g/mL (<i>n</i>) [†]
F1 #91/FC 3	7,146	37.78	746–754 (3)
F1 #91/FC 4	5,857	30.98	731–917 (3)
	Mean	34.38	
CM5 #95/FC 2	19,120	101.14	935 [‡]
CM5 #95/FC 3	18,213	96.33	1,237 [‡]
CM5 #95/FC 4	17,278	91.40	990 \pm 49 (4)
	Mean F1 #95	96.29	
F1 #100/FC 1	6,266	33.15	954 \pm 156 (7)
F1 #100/FC 2	7,174	37.92	1,421 \pm 134 (7)
F1 #100/FC 3	5,250	27.78	1,148 \pm 127 (9)
F1#100/FC 4 [§]	4,767	25.22	1,157 \pm 129 (9)

The flow cells were immobilized with 0.5 mg/mL SEB except for the F1 #91/FC 3 where 1 mg/mL SEB was utilized. In all cases, 30- μ L SEB was injected with a flow rate of 3 μ L/min.

* SEB surface(ng/mm²) = RU/1000 \times 150,000/28,366.

[†] RU of the captured IgG (5 μ g/mL) where (*n*) is the number of analyses at the 5 μ g/mL concentration.

[‡] CM5 #95 FC 2 and FC 3 were evaluated with varying concentrations of IgG, and this single analysis showed capture of IgG at 5 μ g/mL.

[§] F1 #100/FC 4 was modified with 0.25 mg/mL SEB.

surface load in F1 Chip # 91 and F1 Chip #100 was 34.96 ng (three standard deviations [SD]) SEB, while F1 Chip #95 had a mean load of 96 ng SEB. This SEB sensor injected with 5 μ g/mL IgG captured IgG in the range of 935–1237 RU. These results were within the range of the IgG capture of F1 Chip #91 and #100. F1 #100/FC 3 and FC 4 sensors captured similar amounts of SEB IgG with a mean of 1148 and 1158 RU, respectively. These sensor chips were evaluated for their binding to anti-SEB at concentrations of 2.5–50 μ g/mL IgG, and these results are shown in Tables 2 and 3. Table 2 shows the bound anti-SEB RU when injected at varying concentrations of 2.5–500 μ g/mL. Table 3 shows the kinetic properties of anti-SEB. CM5 #95 FC 2 and FC 3 were evaluated 6 months after the preparation of the sensor. These sensors indicated lower affinity constants (10^7 – 10^8) when analyzed with anti-SEB compared with a recently immobilized FC 4 where the anti-SEB had affinity constants of 10^9 – 10^{10} . Anti-SEB captured by F1 #100/FC 1 to FC 4 had a mean affinity constant of 3.35×10^9 (± 0.87 SD). The affinity constant of IgG binding with SEB in FC 4 was within one SD from the mean affinity constant derived from four flow cells. In future studies, we will explore the minimum concentration of SEB in preparation of the sensor surface. It is necessary to be aware of the affinity constants of the binding ligands such that effective

TABLE 2.
BINDING CAPACITY OF CM5 AND PIONEER F1 SEB SENSORS

Chip number/ FC number	Anti-SEB concentration ($\mu\text{g/mL}$)	RU-bound anti-SEB	Chip number/ FC number	RU-bound anti-SEB
F1 #91/FC 3	50	3343	F1 #91/FC 4	3286
	100	3860		3776
	200	4810		4676
	500	6060		5949
	10	1192		1184
	20	NA		1181
	50	2989		NA
CM5 #95/FC 2	12.5	985; 853	CM5 #95/FC 3	603; 704
	25	1152; 1124		900; 1072
	50	1795; 1563		1491; 1494
CM5 #95/FC 4	5	1039; 1020; 910; 990	F1 #100/FC 2	1021
	10	1682; 1513		
F1 #100/FC 1	2.5	1001		
	5	1722		
	10	2437		
F1 #100/FC 3	2.5	986; 921	F1 #100/FC 4	885; 946
	5	1773; 1573		1857; 1606
	10	2483; 2168		
	2.5	986; 921		885; 946
	5	1773; 1573		1857; 1606
	10	2483; 2168		
	2.5	986; 921		885; 946
	5	1773; 1573		1857; 1606
	10	2483; 2168		

A new batch of anti-SEB was used for the evaluation of CM5 #95 and F1 #100 different from those utilized for F1 #91. CM5, FC 2 and FC 3 SEB sensor surfaces were prepared 6 months prior to this antibody evaluation and showed a slightly lower response than the freshly prepared sensors.

TABLE 3.
APPARENT DISSOCIATION RATE, ASSOCIATION RATE AND AFFINITY CONSTANT OF
ANTI-SEB BINDING TO THE SEB SENSOR

Chip number	FC number	Number of analyses	Mean k_d (SD)	Mean k_a (SD)	Mean k_A
CM5 #95	2	6	2.04e^{-4} (1.03)	2.09e^4 (1.20)	1.02e^8
	3	6	2.42e^{-4} (0.29)	1.53e^4 (0.68))	6.3e^7
	4	4	5.02e^{-6} (0.67)	1.23e^5 (0.5)	2.45e^{10}
	4	6	7.22e^{-5} (2.1)	1.52e^4 (1.90)	2.10e^8
F1 #100	1	10	5.69e^{-5} (2.1)	1.17e^4 (0.51)	2.07e^8
	2	10	3.78e^{-5} (0.87)	1.22e^5 (0.47)	3.24e^9
	3	15	3.69e^{-5} (0.51)	1.66e^5 (0.50)	4.50e^9
	4	15	4.51e^{-5} (1.0)	1.62e^5 (0.45)	3.59e^9

The kinetic properties, dissociation rate k_d (S^{-1}) and association rate k_a ($\text{M}^{-1}\text{S}^{-1}$) were determined using the BIAevaluation software using the first-order kinetics: $\text{A} + \text{B} \rightleftharpoons \text{AB}$. The affinity constant k_A (M^{-1}) was determined from the ratio of k_a/k_d while the dissociation constant K_D (M^{-1}) is the reciprocal of K_A .

regeneration reagents can be utilized. A regeneration agent needs to be effective enough to remove the bound IgG without removing the SEB surface. The ligand and antibody with high or low binding (affinity) constants may require different desorption agents. The high residual surface RU shown after the regeneration step of 100 mM HCl may be due to the high affinity ($K_A = 10^9 \text{ M}^{-1}$) and low dissociation ($K_D = 10^{-10} \text{ M}^{-1}$) of the anti-SEB and SEB sensor. In our previous study (Medina 2003), 100 mM HCl was used to regenerate the sensor, but in the recent study, 20 or 30 mM NaOH was necessary to regenerate the sensor without removing the immobilized SEB from the sensor surface. A 10 or 15 mM NaOH concentration was not sufficient to regenerate the sensor, while a 50 mM concentration detached the SEB from the sensor.

Competitive Inhibition Assay in HBS

In earlier studies, a homogeneous assay format with no separation of the bound complex from the free IgG was developed. The results, shown in Fig. 3A, indicated that the SEB was detected from 5–100 ppb with linear responses of $Y = 734.34 - 1.6846 \times (R^2 = 0.964)$ for FC 3 and $Y = 710.68 - 1.7055 \times (R^2 = 0.963)$ for FC 4. In Fig. 3B, the SEB was also detected from 0.78–50 ppb with near linear response at 0–12 ppb after 30-min incubation at room temperature. The flow cells (FC 3 and FC 4) were used in over 75 cycles

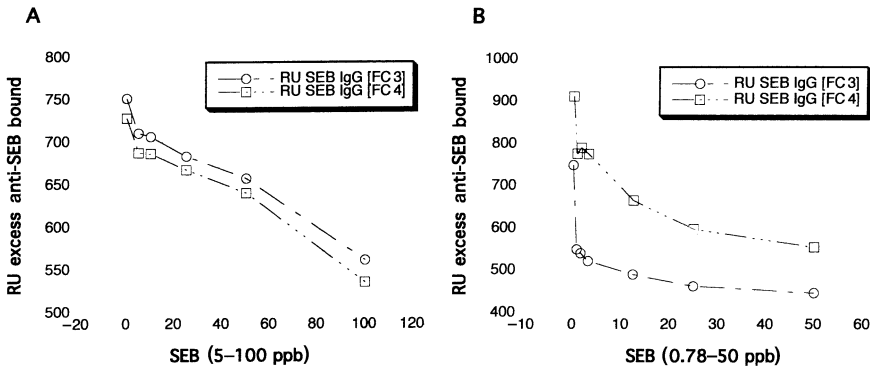


FIG. 3. CAPTURE OF ANTI-SEB IgG BY SEB SENSOR WITHOUT CENTRIFUGATION
 The SEB was allowed to bind with anti-SEB in 200- μL buffer solutions in BIA sample tubes. After incubation, 15 μL was automatically injected into the BIAcore system at 3 $\mu\text{L}/\text{min}$. The IgG-bound RU was measured 6 min postinjection. The IgG bound to the sensor was desorbed with 5 μL 100 mM HCl, and the regenerated surface RU was measured at 1.6 min postinjection. (A) shows the response (RU) of the captured excess IgG versus dose-response of samples containing 0, 5–100 ppb. (B) shows the dose-response of samples containing 0.78–50 ppb SEB. Minimum detection was observed at 0.78 ppb.

of analysis, including the characterization of the anti-SEB binding properties with the SEB sensor. The injection of the SEB samples and anti-SEB mixture in a “homogenous assay” format resulted in high variability of responses at low concentrations, and this variability was presumably due to the binding of the excess unbound SEB IgG and the partially bound IgG~SEB complex to the SEB sensor. As shown in Fig. 1, the SEB sensor may bind to one or two binding sites of the IgG molecule. These results suggested that by separating the bound complex from the free IgG, the assay sensitivity and linearity may be improved. Therefore, the effects of centrifugation at 6000 and 10,000 r.p.m. were studied to determine if these centrifugation speeds would separate the SEB IgG. Our results in the six samples showed a mean 10% difference (gain) in RU binding after centrifugation at 2961 *g*, thus, indicating that the IgG remained in the supernatant. The centrifugation at 10,000 r.p.m. ($8225 \times g$) had a mean loss of 12% in six IgG samples at concentrations of 12.5, 25 and 50 $\mu\text{g/mL}$. These results suggested that the bound IgG~SEB complex can be separated from the free IgG in the supernatant. The auto-injector of the BIAcore system sampled the supernatant and injected the samples over the SEB sensor surface.

An analysis of the SEB samples from 0.78 to 50 ng/mL is also shown in a typical overlay sensorgram of the capture of the excess SEB IgG in HBS (Fig. 4). Anti-SEB IgG (20 μL of 100 $\mu\text{g/mL}$ IgG) was added to 200 μL of SEB from 0.78 to 50 ng/mL in HBS and centrifuged at $2061 \times g$ prior to the BIAcore analysis. The RU responses versus SEB concentration showed linearity from 0 to 3.12 ppb. Beyond this concentration, the SEB IgG binding to the sensor was near saturation. These results showed improvement over the “homogenous assay” format with no separation step. We evaluated the binding properties of anti-SEB (*T*-anti-SEB and *S*-anti-SEB) from two sources, Toxin Technology (Sarasota, FL) and Sigma (St. Louis, MO), respectively. The *T*-anti-SEB generated a higher response at less than 10 ppb SEB, and this antibody was utilized in subsequent studies in milk.

SEB Analysis in Spiked Whole Milk

The milk samples (1 mL) were spiked (inoculated) with 100 μL of 3.125–200 ppb SEB resulting in a final concentration of 0.31–20 ppb. The spiked samples were equilibrated for 30 min or overnight at 4C. The milk samples were heated for 10 min at 95C. The rationale for this heating was to reduce nonspecific binding responses of milk components. Park *et al.* (1992) reported that false-positive results indicating the presence of SE were observed in unheated samples. These false-positive results were significantly reduced when heated for 2 min in boiling water and were not due to the intrinsic peroxidase in the samples. The intrinsic peroxidase in biological

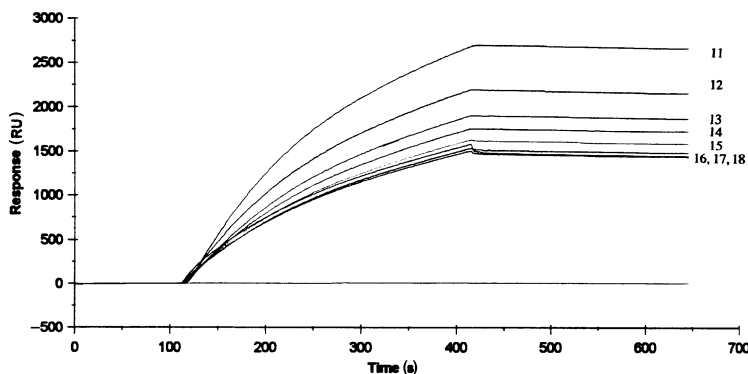


FIG. 4. OVERLAY SENSORGRAMS FOR THE CAPTURE OF ANTI-SEB AFTER CENTRIFUGATION

Injection of excess/unbound SEB (0, 0.78–50 ppb) in HBS after an off line incubation of SEB and anti-SEB in samples followed by centrifugation at 2961 g . The top line is the sensorgram for the “zero” control sample without SEB but contains SEB-IgG, and the next line is a sensorgram for the lowest concentration of added SEB, 0.78 ppb. The bottom line is the sensorgram of HBS (baseline control sample). RU responses of the bound molecules were determined with Report Point at 60 s after the end of anti-SEB injections. The regeneration portion of the sensorgram is not shown.

samples caused an interference in peroxidase enzyme-labeled immunoassays. In our study, milk was centrifuged to separate the SEB from the milk particulates and the lipid fraction where the SEB remained in the fat-free (skim) supernatant. We have reported (Medina 2003) that SEB spiked in ham extracts remained in the supernatant after centrifugation at 2961 g . Bennett and McClure (1980) reported, in a collaborative study on the extraction and separation of SE, that centrifugation at $32,800 \times g$ allowed the SE to remain in the supernatant. Park *et al.* (1994) also separated the SEB from extracts by centrifugation at 16,300 g . Rasooly and Rasooly (1999) and Rasooly (2001) centrifuged the samples at $1000 \times g$ and $14,000 \times g$, respectively, and SEB remained in the supernatant.

Figure 5A shows the dose–response curves when 0.78–25 ppb SEB spiked in milk was plotted against RU of excess and unbound anti-SEB. These dose–response curves indicated changes in the slopes of the lines from different analytical trials of milk spiked with SEB. The mean RUs (SD) in five analyses on the same sensor surface were 1451 (70), 1271 (49), 1256 (56), 1201 (40), 1183 (45), 1123 (30) and 1139 (58) for 0, 0.78, 1.56, 3.12, 6.25, 12.5 and 25 ppb, respectively. With one SD, a sample without SEB generated 1381–1521 RU signals. This suggests minimum detection at 0.78 ppb. One of the reasons for the changing slopes was due to some residual antibody-bound complex on the SEB sensor surface that was not completely removed by the

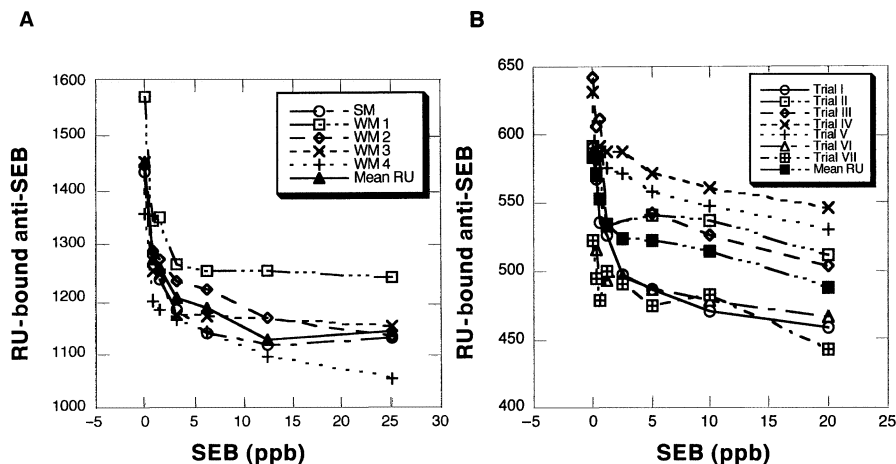


FIG. 5. (A) DOSE-RESPONSE CURVES OF SEB SPIKED IN WHOLE AND SKIM MILK RU responses from the capture of anti-SEB spiked in whole milk (WM) and skim milk (SM). Milk samples were spiked with 0.78–25 ppb SEB, heated at 95°C, centrifuged at 2961 g, incubated with anti-SEB, centrifuged to separate the bound SEB–IgG complex and analyzed with the BIAcore system with a 5-min contact time at 3 μ L/min. The SEB sensor was desorbed with 20 mM NaOH.

(B) DOSE-RESPONSE CURVES OF SEB SPIKED IN SKIM MILK

The RU responses of seven separate analyses were plotted. The mean RU of each dose of SEB is also plotted. Skim milk spiked with 0.315–20 ppb SEB was heated at 95°C for 15 min. Aliquots were transferred to BIAcore sample tubes, adding 20 μ L of 50 μ g/mL anti-SEB and incubated for 1 h. The samples were centrifuged at 2961 \times g and analyzed with the BIAcore system with a contact time of 3 min per sample. The sensor surface was regenerated with 30 mM NaOH.

regeneration agents. This was perhaps due to the high-affinity binding of the antibody to the sensor surface with affinity constant of $K_A = 10^9 \text{ M}^{-1}$ and low dissociation constant of $K_D = 10^{-10} \text{ M}^{-1}$. The increase in RU signals over baseline was not attributed to the milk components because the sensor surfaces were completely regenerated when blank milk samples were analyzed. To improve the sensor surface regeneration, agents were evaluated to select an appropriate agent that could effectively free the SEB sensor from the captured IgG without resulting in the loss of the sensor surface. We have used one to two pulses (1 min each) of 20 mM NaOH to regenerate the SEB sensor surfaces. The use of 100 mM HCl, 10 or 15 mM NaOH and 1 M guanidine-HCl did not completely regenerate the surface, while the use of 50 mM NaOH removed the SEB surface. Due to the changing slopes of the calibration curves, a quantitative determination was not reliable. However, a qualitative detection was possible, i.e., the SEB in the milk samples can be determined by comparing responses to the “zero” milk control containing anti-SEB but no added SEB and the blank milk sample that did not contain SEB or anti-

SEB. Our future research will focus on using antibodies with slightly lower affinity constants or with higher dissociation constants in order to regenerate the sensor surfaces for multiple analyses and to analyze SEB from varied food matrices using the competitive immunoassay format.

SEB Analysis in Spiked Skim Milk

Preparing the skim milk for analysis did not require centrifugation to separate the lipid fraction and other milk particulates. The skim milk samples were spiked with SEB, heated and transferred to the BIAcore tubes. An anti-SEB was added to the milk samples and allowed to bind with the SEB followed by centrifugation to separate the bound complex. As in the whole milk analysis, the supernatant portions were automatically sampled by the BIAcore for analysis of the “free” or unbound anti-SEB. The anti-SEB was injected over the SEB sensor for 3 min at 3 μ L/min. Figure 5B shows the dose–response curves when 0.312–20 ppb SEB spiked in milk was plotted against RU of excess and unbound anti-SEB. As in the analysis of whole milk, these dose–response curves indicated changes in the slopes of the lines. However, the mean RUs (SD) of each dose were 584 (44), 558 (36), 553 (48), 536 (33), 524 (39), 523 (36), 515 (34) and 488 (38) for 0, 0.312, 0.625, 1.25, 2.5, 5.0, 10 and 20 ppb SEB, respectively. To increase the reliability of the interpretation of these results, the RU data were transformed into binding ratio of the sample RU to the “zero” control RU. Table 4 shows the results of 12 replicate analyses, reported as the mean of duplicate analyses in six trials. The

TABLE 4.
 BIACORE ANALYSIS OF SPIKED SKIM MILK RATIO OF SAMPLE BINDING TO ZERO
 CONTROL (SAMPLE WITH ANTI-SEB ONLY)

SEB in sample	I*	II	III	IV	V3.2	VI	Mean	Standard deviation
HBS	1.29 [†]	2.3	1.04	0.6	0.65	0.65		
Milk blank	6.45	8.9	19.7	6.05	14.4	4.8		
0 ppb	100	100	100	100	100	100	100	
0.312	90.8	95.2	95.3	95.9	94.4	95.8	94.58	1.9
0.625	86.2	90.2	97.1	95.5	95.6	95.6	93.36	3.8
1.25	81.8	88.0	86.3	91.8	95.3	96.0	89.87	5.0
2.50	78.2	82.5	96	90.2	94.8	94.2	89.32	6.7
5.00	78.1	80.8	99.2	89.2	92.6	92.4	86.88	5.5
10.00	72.5	78.4	86.2	87.3	90.9	90.6	84.32	6.7
20.00	67.9	76.4	82.4	87.1	84.9	88.6	81.22	7.1

* Mean of duplicate analyses in six trials.
[†] Percent binding ratio: RU binding of sample/RU binding “0” sample with anti-SEB but no toxin \times 100.

results show the reduction in binding of the free (unbound) anti-SEB when SEB is present in the sample. Samples spiked with 0.312 ppb were detected with one SD of 94.58% (± 1.76), suggesting a limit of detection (LOD) at this concentration. Samples spiked with 1.25 ppb were detected with two SD (94.58% ± 2.8) and indicated that a 1.25-ppb sample was detected with greater certainty.

CONCLUSION

The current study showed that a competitive immunoassay using the BIAcore SPR biosensor can be used for sensitive detection of SEB. We have optimized the sample preparation for the biosensor analysis of milk samples. The milk samples were preheated and centrifuged at $2961 \times g$ to reduce interference. The competitive immunoassay also consisted of separating the bound complex from the free IgG by centrifugation at $2961 \times g$. This assay format resulted in the detection of SEB from 0.312 to 20 ng/mL in spiked milk. However, SEB from the unknown samples was qualitatively determined by comparing the responses to milk control samples with anti-SEB but without SEB. Using this approach, a minimum of 0.31 ng/mL SEB was detected in whole and skim milk. The sample preparation required less than 1 h for 20 samples, and the BIAcore analysis was completed in 15 min per sample or four samples per hour. This assay is more rapid than our previously reported sandwich assay procedure with a biosensor analysis of 25 min per sample. The biosensor analysis is fully automated, and it is anticipated that the SPR biosensor method will be extended to detect other toxins in various food matrices if suitable antibodies are available.

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